

The University of Chicago

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MODIUM GALLINACEUM AND
PLASMODIUM LOPHURAE

A DISSERTATION SUBMITTED TO THE FACULTY OF THE
DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

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IN VITRO OPSONIC TESTS WITH *PLASMODIUM GALLINACEUM* AND *PLASMODIUM LOPHURAE*

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Repeated attempts have been made to determine whether circulating antibodies appear in the blood of animals infected with malarial parasites. Thus, one species or another of *Plasmodium* has been used in protection tests, agglutination and precipitation tests, skin tests and complement-fixing reactions. Since every technic has yielded some positive results, it is possible that inadequate technics are responsible for the negative results. Immunological reactions in malaria are of varying degrees of specificity. Complement fixation and precipitin tests are relatively nonspecific, apparently because most of the antigens involved are common to many species of *Plasmodium*. Immunity to superinfection, agglutination and protection tests are more specific and, in the primate malarias, may be highly strain-specific.

It is unnecessary to labor the point that phagocytosis is the controlling factor in acquired immunity to malarial infections. Blockade of the reticulo-endothelial system in avian malaria was shown by Kritschewski and Meerson (1932) to interfere with the immune mechanism against such infections.

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Gingrich (1941) found that although blockade did not affect the natural immunity of birds to *P. relictum* and *P. cathemerium*, it radically disturbed the development of acquired immunity. Trager (1941) arrived at essentially the same conclusion for *P. lophurae*. The extensive studies of Cannon and Taliaferro (1931), of Taliaferro and Cannon (1936) and of Taliaferro and Mulligan (1937) clearly proved that, in avian and simian malaria, phagocytosis of the plasmodia by the cells of the lymphoid-macrophage system plays the decisive role in the mechanism of immunity.

The mechanism of acquired immunity to malaria is generally considered to involve a specific immune factor which prepares the malarial plasmodia or the parasite-erythrocyte combination for phagocytosis by the macrophages of the host. Though such a specific opsonin has not been demonstrated, it has been postulated by many investigators. Ziemann (1924) wrote that the serum of untreated chronic malaria patients probably contains more opsonins than does normal serum. Taliaferro (1932) suggested that such opsonins, locally formed, may be present in the peripheral blood in a concentration too low to be demonstrated by the technics so far employed. Taliaferro and Cannon (1936) stated that an opsonin, complementing the cellular phagocytic activity, is the logical agent in malarial immunity. Coggeshall and Kumm (1937) believed that the protection of monkeys against *P. knowlesi* and *P. inui* is due to an opsonin. Gingrich (1941) stated that that in avian malaria the humoral factor aiding the phagocytic activity of

the macrophages is probably an opsonin, and Coggeshall (1941) wrote that protective antibody probably sensitized the parasite and thereby made it more susceptible to phagocytosis.

Euglobulins found by Brown (1933) in immune bird malaria serum lowered the electric charge of homologous erythrocytes and thus stimulated the phagocytosis of these cells. This reaction, however, was not specific, as the euglobulin could be absorbed by mouse erythrocytes and even by bacteria, with the result that such material, as well as the homologous erythrocytes, were phagocytosed with increased vigor. The fact, recorded by Findlay and Brown (1934), that in avian malaria the incubation period was longer when parasites from the end of an infection were used as the inoculum than when parasites from the beginning of an infection were used suggests that the post-crisis parasites were opsonized in vivo.

The present paper describes the occurrence of circulating opsonins in the serums of chickens hyperimmunized against *P. gallinaceum* and *P. lophurae*. Their presence was demonstrated by an opsonic technic using as phagocytes macrophages derived from blood agranulocytes. The use of macrophages rather than of circulating granulocytes was considered rational since the in vivo phagocytosis of plasmodia has been shown to involve primarily the cells of the lymphoid-macrophage system (vide ante). Spleen explants were tried at first, but were discontinued because the number and age of the macrophages, their distribution in the culture, and the "micro"-conditions of their exposure to the parasitized cells were all variables which could not be controlled. Blood agranulocytes proved more satisfactory because a homogeneous growth of the phagocytes could be regularly obtained. The use of macrophages derived from

blood leucocytes is in line with the opinion of Taliaferro (1941) that "the lymphocyte is probably the single most important source of new macrophages in malarial immunity. . . . By far the majority of new macrophages arise from lymphocytes with or without passing through an intermediate monocyte stage."

MATERIALS AND METHODS

Briefly, the opsonic test consists of:

Growing macrophages from blood agranulocytes (lymphocytes and monocytes) in vitro in a flask, whose base has been removed and in its place a coverslip has been cemented.

Adding antigen and test serum to the macrophage culture.

Incubating the culture at 37 C for varying lengths of time.

Fixing the culture containing the macrophage preparation in situ.

Removing the coverslip from the flask and staining and mounting it.

Estimating the amount of phagocytosis per macrophage per culture by microscopic examination of the coverslip preparation.

Aseptic precautions were observed throughout all manipulations of the culture. Tyrode's solution without CaCl_2 was found to be a satisfactory washing fluid and will be referred to as modified Tyrode's solution. CaCl_2 was omitted to prevent clotting of the leucocytes and thereby to obtain a homogeneous suspension.

The flask was prepared as follows. The base was removed from a standard Carrel flask (diameter = 3 cm) by a glass blower, and the rim of the side wall of the flask was ground flat so that a tight seal could be obtained when the bottomless flask rested on a plane surface. A plastic cement, Gelva V-7 in acetone (Shawinigan Products Company, New York City), was used to join the bottomless flask to a 40 mm by 40 mm number 1 coverslip. The rim of the flask was brushed with a solution of 15 gm of Gelva powder in 85 ml of acetone and gently pressed down upon the surface of a coverslip. A thicker solution of Gelva, 30 gm in 70 ml of acetone, was liberally brushed into the angle between the vertical wall of the flask and the coverslip. When the cement had set, the flask was plugged with cotton and sterilized by dry heat. Unless an inadequate amount of cement was used, the flask was watertight. A similar technic for the cementing of coverslips to roller tubes was described by Shaw

et al. (1940). After all the culturing manipulations were completed, the cement was dissolved in 75% alcohol and the coverslip containing the culture material could then be treated as a whole mount preparation.

The following serums were used in each test:

1. Serum as a medium in which the agranulocytes hypertrophied into macrophages.

2. Serum as an opsonizing agent.

All chicken or rabbit serum for the tests was collected from blood drawn aseptically by cardiac puncture from animals which were previously starved for 24 hours so that the serum was free of visible fat. The technic of Lewis (1928) for the cardiac puncture of chickens was followed except that the needle was inserted through the angle of the sternum and not from the side. Pooled sterile normal serum was used as a culture medium for the hypertrophy of agranulocytes into macrophages. It could be used fresh or after storage for varying lengths of time up to several months at 6 C. A precipitate, which generally appeared in stored serum after about a week, could be sedimented by centrifugation and discarded.

The opsonizing serums were obtained during the course of the various infections studied. Immune serum was obtained during initial infections after the crisis, in most cases when circulating parasites were no longer found. Hyperimmune serums were obtained after from 2 to 7 superinfections.

In superinfecting chickens to obtain hyperimmune serum, such quantities of parasitized blood were inoculated that 100-700 parasites could be found among 10,000 red cells immediately after the injections.

Pneumococci, or citrated red blood cells heavily infected with *P. gallinaceum* or *P. lophurae*, were obtained aseptically and washed twice with modified Tyrode's solution just before use.

Macrophages for the opsonic test were grown from normal agranulocytes obtained as follows. Blood was drawn from animals into 4% sodium citrate in physiological saline at the rate of 5 ml of blood to 1 ml of citrate. The citrated blood was collected in 50 ml cork-stoppered sterile centrifuge tubes, was immediately chilled in an ice-bath and was centrifuged at 2,000 rpm for 5 minutes. After most of the supernatant citrated plasma was discarded, the buffy coat layer from about 30 ml of blood, together with the plasma left above it, was thoroughly mixed with 10 ml of modified Tyrode's solution and recentrifuged. After a second similar washing, the buffy coat layer, containing as few red cells as possible, was mixed with normal serum and was distributed to

the flasks. Since, on the one hand, it was expedient to have a single layer of macrophages growing on the coverslip surface rather than many superimposed layers, and since, on the other hand, it was convenient to have no fewer than 10-20 macrophages in an oil-immersion field when making the phagocytic count, the best proportion of buffy coat cells to serum was found to be as follows: the buffy coat from 2.5 ml of chicken blood or from 5 ml of rabbit blood suspended in 0.6 ml of normal serum. The inoculum for an entire experiment was prepared at one time and was frequently agitated to insure homogeneity while filling the flasks. The flasks, after receiving their complement of buffy coat and serum, were promptly rotated to distribute the cells, and were tightly corked and placed on cardboard trays at 37 C for 24 hours. At this time, the lymphocytes and monocytes of the washed buffy coat inoculum had developed into large macrophages with abundant cytoplasm and many of them were firmly attached to the coverslip base of the culture. They were then ready to be exposed to a suspension of antigen and serum for the opsonic test.

To examine cultures microscopically with an oil-immersion lens, the Carrel flask, supported bottom side up in a small cylinder notched at one point to accommodate the arm of the flask, was placed on a microscope stage, and transmitted light was focussed at the level of the coverslip, i.e., 28 mm above the normal focal plane by an accessory condenser.

After the supernatant medium, containing exhausted serum, free blood cells and metabolites had been removed, each culture was washed in 2-3 ml of fresh modified Tyrode's solution at 37 C. As soon as the washing fluid was aspirated, antigen and test serum were rapidly added to avoid desiccation of the washed macrophages. The flasks were rotated to distribute their contents evenly over the macrophages, corked and incubated at 37 C for periods of time varying with the nature of the test.

Subsequently, the supernatant suspension was drawn off, and the culture was washed with modified Tyrode's solution. Immediately thereafter, before the culture could dry, the flask was filled with Zenker-formol fixative (9 parts of Zenker's fluid mixed just before use with 1 part of neutralized formalin) and was placed in a rack for 5 minutes, coverslip side up so that no precipitate could collect on the culture. The fixative was then poured off and the interior of the flask, after being rinsed several times with tap water until the rinsing fluid was no longer yellow, was filled with 50% alcohol containing enough tinc-

ture of iodine to give it a sherry color. In a few minutes the iodine-alcohol was discarded, and the flask was rinsed in 75% alcohol, filled with 75% alcohol and placed in a Petri dish containing 75% alcohol overnight to dissolve the Gelva cement.

When the cement had dissolved and the bottomless flask had been lifted off, the coverslip bearing the whole-mount preparation was put into stain after being carefully washed 2 to 3 minutes in each of the following solutions:

- 1) 3 changes of 75% alcohol.
- 2) 2 changes of 50% alcohol.
- 3) 2 changes of distilled water.

All preparations were stained according to the following modification of Maximow's hematoxylin-eosin-azure II technic. The coverslip was floated overnight, culture side down, on a solution of Delafield's hematoxylin (1 drop of stock solution in 50 ml of distilled water for chicken macrophages or 1 drop of stock solution in 100 ml of distilled water for rabbit macrophages). The following morning the culture was floated on tap water in a Petri dish for several hours or until the stain turned from lavender or gray to blue. It was then floated overnight on a solution containing:

- 1) 30 ml of 0.1% eosin solution (water-soluble yellow) in distilled water.
- 2) 100 ml distilled water.
- 3) 15 ml of 0.1% azure II solution in distilled water.

These materials were mixed in the order named just before use. The next morning the preparation was differentiated and dehydrated by being passed through the following reagents:

- 1) distilled water, a few seconds.
- 2) 95% alcohol, 2 changes. A preparation was left in the second change of 95% alcohol until nuclear detail could be seen in the stained cells as determined by microscopic examination.
- 3) Absolute alcohol, 3 changes, 2 to 3 minutes in each.
- 4) xylol, 2 changes, 2 to 3 minutes in each.

When a preparation had cleared, it was mounted in a thin solution of clarite in xylol on a 2×3 inch slide.

EXPERIMENTAL DATA

A. Cells found in cultures prepared from the buffy coat from chickens' blood

The blood elements appeared as follows in cultures which were examined microscopically in the living condition or after incubating for 1, 2, 3, 4, 22, 27,

31, 46 or 50 hours, and fixing and staining:

In the circulating blood the lymphocytes and monocytes were roughly spherical in shape and had little cytoplasm. During the first few hours of incubation, these cells crept about in a culture, and many of them flattened and developed a quantity of vacuolated cytoplasm with threadlike pseudopods. Their nuclei assumed a flattened, irregularly oval shape, while their chromatin masses, becoming smaller and more widely spaced, stained more lightly. By the fourth hour of incubation, practically all of the blood agranulocytes had changed to polyblasts about the size of a chicken erythrocyte.

With continued incubation the polyblasts grew rapidly until in a 22-hour culture they had developed into typical macrophages, each consisting of abundant, foamy cytoplasm and a large, irregular nucleus with delicate chromatin granules and 1 or 2 conspicuous nucleoli (essentially similar to Fig. 8 in Plate 1). In some, the cytoplasm had definite boundaries (Fig. 5, Plate 1) whereas, in others (Fig. 8, 9, 11, 12, Plate 1), it faded away at the edges into hardly visible membranes or pseudopods. It normally contained numerous lipid droplets. Developed macrophages tended to stick to the coverslip more tenaciously than young forms, as determined by their prevalence in the flasks. The macrophages mechanically supported themselves on threads of fibrin or lint when such material was present and assumed spindle-like shapes. Such spindle-shaped macrophages were also regularly seen at the margin of the cultures where the necessary mechanical support was provided by the vertical wall of the Carrel flask. The diameter of 24-hr. macrophages varied from 10 to 30 μ , and differences in the depth of stain taken up by different cells sug-

gested that cell volumes varied as well. Macrophages undergoing mitosis were occasionally seen. Such cells were not actively phagocytic, but occasionally contained a few pigment granules.

A few of the polyblasts were phagocytic in the 3- and 4-hour cultures. For 22 hours phagocytosis increased in intensity and, thereafter, was marked. Although blood cells of all types were taken up and digested by the macrophages, thrombocytes were ingested most regularly in the 22-hour cultures and erythrocytes in the 27-hour to 50-hour cultures. This finding illustrates the scavenging action of the macrophages since degenerating thrombocytes with pyknotic nuclei were seen for the first time in the 22-hour cultures and vacuolated erythrocytes first appeared in the 27-hour cultures. Degenerating lymphocytes, monocytes and granulocytes were rarely found but were occasionally seen within macrophages.

Heterophil leucocytes were seen in all cultures. Some were compact and spherical; others were flattened out with blunt extruded pseudopods. Heterophils increased somewhat in size, but were rarely larger than erythrocytes. Heterophils, as well as macrophages, were actively phagocytic *in vitro*.

Eosinophils were rarely seen.

Chicken buffy coat cultures always contained numerous thrombocytes. In 1-hour cultures they formed compact masses of several hundred; but later, due to the movement of the cells, formed looser and less compact clusters. Many leucocytes were found in or near these clusters.

In 1-hour cultures many thrombocytes retained the form they have in the circulating blood, that of a bluntly oval cell with a sharp outline, somewhat smaller than an erythrocyte, containing when stained a deep purple nucleus and a characteristic pink granule in the

vacuolated cytoplasm. Others had assumed a stellate form with blunt or lacy pseudopods and a flatter, less dense nucleus, which stained a robin's egg blue and might have an area twice that of the nucleus of the circulating thrombocyte. The pink cytoplasmic granule was still recognizable and persisted through the 50-hour cultures. In 22-hour cultures, many oval thrombocytes had pyknotic nuclei and assumed bizarre degenerating shapes, whereas only a few of the amoeboid thrombocytes degenerated. As mentioned previously, the degenerating thrombocytes were actively taken up by macrophages.

All cultures contained a few red blood cells. Vacuolated red cells appeared for the first time in 27-hour cultures, and thereafter were found in all stages of digestion in the macrophages.

B. The opsonic test with pneumococci and test antiserum

1. *Experiment 1.*—Before investigating the possible presence of a circulating opsonin in avian malaria, it was essential to test the ability of blood buffy coat macrophages to phagocytose sensitized organisms whose behavior was well known in other opsonic tests.

A strain of Type 1 pneumococcus, used by Dr. O. H. Robertson in studying the phagocytosis of pneumococci by the circulating leucocytes of dogs and rabbits, was obtained. Eighteen-hour cultures of this organism in veal infusion dextrose broth were used as the test antigen.

High titer therapeutic antipneumococcus serum was obtained from Lederle & Co. (antipneumococcus rabbit serum, Type I, containing 50,000 units in 5 ml, and preserved with 0.4% phenol and 1:50,000 phenyl mercuric acetate).

Normal fresh rabbit serum was used in the normal control cultures.

As suggested by O. H. Robertson (personal communication), the serums were diluted 1:1,000 since lower dilutions were likely to agglutinate the pneumococci and thereby interfere with phagocytosis. The dilutions were prepared in modified Tyrode's solution. Half of them were

used fresh and the other half after inactivation at 55 C for 30 minutes.

Twenty-four-hour cultures of rabbit and chicken macrophages were used, although at this time the mean size of the chicken macrophages was larger. To each of these were added 0.55 ml of the serum, diluted 1:1,000, and 0.05 ml of an 18-hour culture of Type I pneumococcus. The flasks were then treated as previously described, after a 1-hour incubation period.

As may be seen in Table 1, the macrophages in cultures 1, 2, 3 and 4, which contained normal serum, phago-

Quellung reaction. In fact, the swelling was so marked in the chicken macrophage preparations that clumps of ingested pneumococci seemed to be embedded in a hyaline matrix (Fig. 6 and 7, Plate 1).

Examination of Table 1 and Figures 1 through 7 of Plate 1 indicates that chicken and rabbit buffy coat macrophages acted similarly. Thus, neither kind phagocytosed pneumococci to any marked extent in the presence of normal

TABLE 1.—*In vitro* phagocytosis and agglutination in opsonic cultures with pneumococci and test antiserum.

Culture number c: chicken r: rabbit	Test serum	Treatment of serum	Phagocytosis of pneumococci by macrophages	Agglutination of pneumococci
1c	normal	fresh	minimal	none
2c	normal	inactivated	minimal	none
3r	normal	fresh	minimal	none
4r	normal	inactivated	minimal	none
5c	immune	fresh	extensive	extensive
6c	immune	inactivated	extensive	extensive
7r	immune	fresh	extensive	extensive
8r	immune	inactivated	extensive	extensive

cytosed only an occasional pneumococcus (Fig. 1 and 5, Plate 1). The macrophages in flasks 5, 6, 7 and 8, on the other hand, which contained immune serum, had phagocytosed numerous pneumococci and were actively digesting them, as shown by the fact that some of the ingested cocci were imperfectly stained, vacuolated, irregular in shape or shrunken (Fig. 2, 3, 4, 6 and 7, Plate 1). Furthermore, some macrophages which were packed with blood cell debris were able to take up opsonized pneumococci as well.

In addition to phagocytosis, agglutination of the uningested pneumococci was always seen in immune serum (Fig. 4 and 7, Plate 1). The clumps of bacteria tended to localize around the macrophages in streamer-like masses. Furthermore, capsular swelling of the pneumococci taken up by chicken macrophages was seen in the homologous immune serum, the so-called Neufeld

serum, whereas both vigorously phagocytosed pneumococci in the presence of opsonin. Therefore, a test using chicken buffy coat macrophages should reveal the presence or absence of circulating opsonin in the serum of chickens hyperimmunized against *P. lophurae* or *P. gallinaceum*.

Since the foregoing immune reactions took place as vigorously in the flasks containing inactivated immune serum as in those containing fresh immune serum, complement seemed unnecessary. This conclusion was corroborated by the findings in a series of cultures similar to those described in the preceding section, to each of which was added 0.2 ml of a 1:10 dilution of fresh pooled guinea pig serum. This is in accord with the results of Bloom (1927) on the phagocytosis of opsonized pigeon red cells by rabbit lung macrophages. He found that inactivated immune serum was as potent as untreated immune serum in

stimulating phagocytosis of the test antigen.

C. Standardization of the opsonic test for avian malaria

1. *Source of macrophages and of test serum: experiment 2.*—To study the cellular elements in the opsonic test, macrophages were grown from agranulocytes of a normal chicken and from those of a chicken superinfected twice

accommodate a dozen cultures at a time, but small enough to be used in the laboratory incubator. The platform was hinged to a fixed base along one of its sides and the alternate raising and lowering of its free side through an arc of about 2.75 inches gave an oscillating motion to the cultures mounted on it. The motor was so adjusted that the rate of oscillation could be varied and periods of oscillation could be alternated with periods of rest.

The effects of 3 types of oscillation with non-oscillated controls were tested on the 4-hour period of phagocytic activity as follows: (1) con-

TABLE 2.—Exp. 2. *Phagocytic indices in opsonic cultures (macrophages from leucocytes from a hyperimmune and a normal chicken) with P. gallinaceum and hyperimmune or normal test serum.*

Culture number	Source of leucocytes	Test serum	Phagocytic index	Mean
10	Hyperimmune chicken	Hyperimmune	1.74	2.09 ± 0.05*
11	Hyperimmune chicken	Hyperimmune	2.45	
12	Hyperimmune chicken	Normal	0.99	1.23 ± 0.05
13	Hyperimmune chicken	Normal	1.48	
14	Normal chicken	Hyperimmune	1.65	2.30 ± 0.05
15	Normal chicken	Hyperimmune	2.95	
16	Normal chicken	Normal	1.32	1.13 ± 0.04
17	Normal chicken	Normal	0.94	

* Standard error.

with *P. gallinaceum*. These were then tested in normal serum and in serum from a chicken superinfected 7 times with *P. gallinaceum*. In the in vitro cultures, using the technic previously described, the macrophages, regardless of their source, reacted essentially similarly when tested with hyperimmune or with normal serum, as may be seen by the results in Table 2, whereas the hyperimmune serum doubled the phagocytic index over normal serum.

On the basis of the results of this experiment, all macrophage cultures in later experiments were grown from leucocytes collected from normal chickens, whereas test serums from immunized and normal birds were studied in detail.

2. *Oscillation: experiment 3.*—This experiment was undertaken to determine whether oscillation would affect the phagocytic indices (see Table 4) of macrophages.

A platform 8×11 inches oscillated by an electric motor, was used. It was large enough to

stant oscillation at 37 complete excursions per minute which simulated the rapid flow of blood in the larger blood vessels; (2) constant oscillation at 12 excursions per minute simulating sluggish blood flow; and (3) 5 minutes of oscillation at 12 excursions per minute alternated with 15 minutes at rest, which simulated the sluggish and intermittent flow in the spleen sinuses. During the 24-hour growth of the macrophages and 20-hour digestion period, the cultures were at rest. In Parts I and II, normal serum was used, and in Part III, normal and hyperimmune serums were used. Parts I, II and III were done at different times and with different macrophages, and may not, therefore, be compared with one another.

The results of this experiment are summarized in Table 3.

The higher phagocytic indices of the resting as compared to the oscillated cultures in Part I indicate that rapid and constant oscillation interfered with phagocytosis. This supports the commonly accepted belief that little phagocytosis by blood phagocytes takes place in the circulating blood while it is traveling rapidly through the larger blood vessels.

In Parts II and III there was no statistically significant difference between the control cultures exposed to parasitized cells suspended in normal serum and those which were constantly oscillated or intermittently oscillated at a slow rate. On the other hand, in Part III, the cultures in which the parasitized cells were oscillated in hyperimmune serum were significantly less

well before the crisis because a circulating opsonin, if it occurred, might be present around the time of the crisis and thus opsonize the antigen. The sediment of washed cells was generally suspended in twice its volume of modified Tyrode's solution after centrifuging for 5 minutes at 2,000 rpm. Roughly 20 to 100 million parasites were introduced per flask. Antigen was always freshly prepared on the day the test was done.

4. *Incubation of the culture.*—When the malarial antigen and test serum were added to the 24-hour macrophage culture, it was found that

TABLE 3.—Exp. 3. *Phagocytic indices in opsonic cultures oscillated while P. gallinaceum was in contact with hyperimmune or normal test serum.*

	Culture number	Oscillation	Test serum	Phagocytic index	Mean
Part I	18	Rapid*	Normal	0.37	0.40 ± 0.02
	19	Rapid	Normal	0.43	
	20	None	Normal	0.67	0.65 ± 0.03
	21	None	Normal	0.63	
Part II	22	Slow**	Normal	0.21	0.23 ± 0.01
	23	Slow	Normal	0.31	
	24	Slow	Normal	0.15	
	25	None	Normal	0.32	0.26 ± 0.02
	26	None	Normal	0.20	
	27	Intermittent***	Hyperimmune	0.39	0.31 ± 0.01
Part III	28	Intermittent	Hyperimmune	0.24	
	29	None	Hyperimmune	0.43	0.43 ± 0.01
	30	Intermittent***	Normal	0.07	0.09 ± 0.006
	31	Intermittent	Normal	0.11	
	32	None	Normal	0.12	0.10 ± 0.007
	33	None	Normal	0.08	

* 37 excursions/minute.

** 12 excursions/minute.

*** 12 excursions/minute for 5 minutes; at rest for 15 minutes.

phagocytic than similar cultures at rest, although all hyperimmune cultures were significantly more phagocytic than their normal controls. Oscillation may have interfered with the adhesion of the opsonized blood cells to the macrophages and to the coverslip (vide infra).

Since oscillation was not more effective than rest and actually depressed phagocytic activity in the hyperimmune serum of Part III, cultures in later experiments were not oscillated.

3. *The antigen.*—The antigen used in determining the opsonin-content of *P. gallinaceum* and *P. lophurae* antisera was a suspension of thoroughly washed blood cells drawn from a chicken during the initial acute rise of its infection at a time when there were 30 to 60 homologous parasites per 100 red blood cells. The blood was taken

the opsonic titers of the antisera were lower than those with antipneumococcus antisera. Therefore, the antigen and test serum were incubated together for 4 hours instead of 1 hour. However, at the end of this 4 hour period, the macrophages were often too engorged to estimate accurately the number of parasites they contained (see Fig. 13, Plate 1). This difficulty was overcome by drawing off the supernatant fluid from the culture, washing the culture with warm (37 C) modified Tyrode's solution, adding 0.6 ml of normal serum and reincubating for 20 hours. The contents of the flask were then rinsed in modified Tyrode's solution and fixed and stained as previously described. During the 20-hour reincubation, the macrophages digested practically all the material they had phagocytosed except the pigment of the parasite (Fig. 11, Plate 1). This pigment was used as an index of the phagocytic activity of the macrophages. Although a certain amount of free parasite pigment occurred in every antigen sample, its uptake was assumed to

be uniform since equal quantities were presented to all cultures in a given series.

5. *The phagocytic index.*—The microscopic examination of macrophages was begun just within the outer edge of the widest diameter of a given culture and continued through adjacent oil immersion fields toward the center. Those at the extreme outer edge were not included because they grew beneath the wall of the Carrel flask and had, therefore, not been exposed to the suspension of blood cells in serum. Every complete macrophage was examined in every microscopic field containing between 10 and 20 macrophages. Fields containing more than 20 or less than 10 complete macrophages were usually disregarded in order to avoid the irregularities introduced by extreme crowding or sparseness of the phagocytes.

TABLE 4.—*Number of pigment clumps in 500 macrophages and the resultant phagocytic index, i.e., pigment clumps per macrophage, in a standard opsonic culture with P. gallinaceum and normal test serum.*

Number of pigment clumps	Macrophages classified as to pigment content	Total number of pigment clumps in all macrophages	Phagocytic index
0	90	0	
0.15	192	28.8	
0.4	85	34.0	
1	107	107	
2	15	30	
3	11	33	
Total	500	232.8	0.47 ± 0.03

The amount of pigment in each of 500 macrophages per culture was ascertained and was tabulated as parts or multiples of a "pigment clump." A "pigment clump" was arbitrarily designated as the equivalent of 20 pigment granules, and its size was approximately that of the mass of pigment seen in an erythrocyte containing a fully developed asexual segmenter of *P. lophurae*.

A macrophage containing no pigment was called "negative" although it may have phagocytosed material such as blood cell debris and young parasites containing no malarial pigment. All macrophages containing 1 to 5 granules of pigment were designated "+," i.e., 0.15 pigment clump. Those containing 6 through 10 granules were designated 0.4 pigment clumps. Those containing larger quantities of pigment were designated 1 clump, 2 clumps, etc., depending on the amount estimated. "One clump," for instance, was judged to be within 0.6 to 1.5 clumps. Thus, the cell depicted in Fig. 9, Plate 1, contains the

equivalent of about 1.2 clumps and would fall in the category of "1 clump."

The phagocytic index of any culture was considered to be the mean number of pigment clumps phagocytosed per macrophage in a random sample of 500 macrophages. The way in which the data were collected and the resultant phagocytic index obtained may be seen in Table 4. The macrophages in this culture were exposed to *P. gallinaceum* in normal serum and had a phagocytic index of 0.47 ± 0.03 .

Standard errors were calculated for all large groups of indices.

That macrophages should phagocytose a certain amount of material in normal serum was to be expected since one of their physiological functions is scavenging in vivo. Such phagocytosis is analogous to the sluggish phagocytosis of precrisis natural immunity in vivo, as described for *P. brasilianum* by Taliaferro and Cannon (1936). Macrophages in normal serum generally tended to ingest free pigment clumps (Fig. 12, Plate 1), whereas those in hyperimmune serum tended to ingest, in addition, whole parasites, the entire parasitized red cell combination and whole red cells (Fig. 13, Plate 1). Thus, the macrophages in normal serum exhibited a scavenging activity, whereas those in hyperimmune serum reacted as if stimulated.

6. *Adhesion phenomena.*—Cultures exposed to hyperimmune serum or plasma in general differed from those exposed to normal serum or plasma not only in their phagocytic indices, but in 3 other respects as well, viz., agglutination, adhesion and rosette formation.

Agglutination occurred after 10 minutes at 37 C. Normal and parasitized red cells in hyperimmune serum formed large clumps which collected in the supernatant fluid and were clearly visible to the naked eye. Indeed, this massive agglutination probably interfered with phagocytosis at times by making the opsonized antigen less available to the macrophages. Such an inter-

ference has been observed in pneumococcus opsonization by Robertson (personal communication).

No agglutination was ever observed when washed parasitized cells were suspended in normal serum or plasma.

Opsonized red cells after exposure to hyperimmune serum appeared to be particularly sticky for they adhered to the coverslip so tenaciously that even vigorous rinsing with modified Tyrode's solution did not loosen them. Both normal and parasitized red cells took part in this reaction. Red cells exposed to normal serum, on the other hand, only occasionally adhered to the coverslip.

Many macrophages, after a short period of incubation with parasitized blood in hyperimmune serum, were surrounded by normal and parasitized erythrocytes radially arranged in the form of a nap or pile (see Fig. 13, Plate 1, for a partial rosette). These rosettes were sometimes so dense that the macrophage was almost completely hidden. They occurred around macrophages in every stage of phagocytic activity. Similar rosettes have been described by Bloom (1927) in cultures of normal rabbit lung macrophages exposed to pigeon erythrocytes, in which the pigeon cells adhered radially to the lung macrophages. The adhering cells were promptly ingested when immune antipigeon erythrocyte rabbit serum was added to the lung cultures. Agglutination *in vivo* of red cells infected with *P. falciparum* has been observed by several investigators (see review in Cannon, 1941). McLay (1922) and Thomson (1924) have also described the adhesion of red cells infected with the same species to endothelial cells (= macrophages) in cultures and in the body. Mudd and his coworkers (1934) have stressed the point that adhesion of the opsonized antigen to the phagocyte is a regular preliminary stage to ingestion.

Since the adhesion phenomena were regularly seen together and express the same change in the surface properties of the opsonized cells, viz., an increased stickiness, they all probably represent the action of a single antibody or complex of antibodies. Furthermore, since a raised phagocytic index was always accompanied by these surface changes in the opsonized cells, although the reverse was not true—probably due to the fact that excessive agglutination masked the action of opsonin—there is presumptive evidence that opsonization and the adhesion phenomena are due to the same antibody. This statement agrees with the conclusions of Mudd *et al.* (1934) that "specific precipitation, agglutination, surface- and phagocytosis-promoting-effects of serum are all consequences of one underlying phenomenon."

7. *The standard malaria opsonic test.*—In consideration of the preceding facts in this section and in Materials and Methods, the standard malaria opsonic test and the determination of the result consisted of the following:

Growing macrophages on the coverslip base of a modified Carrel flask from chicken blood agranulocytes in normal, unactivated, undiluted chicken serum in the proportion of buffy coat from 2.5 ml chicken blood per 0.6 ml normal serum.

Incubating the culture at 37 C for 24 hours.

Washing with modified Tyrode's solution and immediately adding (1) 0.05 ml washed parasitized red cells suspended in modified Tyrode's solution, which had been collected from a chicken during the acute rise of its infection and (2) 0.55 ml undiluted unactivated test serum (stored for varying periods of time at 6 C).

Immediately shaking the culture for a few seconds.

Incubating the culture at 37 C for 4 hours.

Examining the culture microscopically at 1 hour for adhesion phenomena and examining the supernatant fluid at the end of the 4-hour period for agglutination.

Washing the culture with warm modified Tyrode's solution, adding 0.6 ml of normal, undiluted chicken serum and reincubating for 20 hours.

To this point, sterile precautions had to be maintained.

Fixing the culture on the coverslip in situ.

Removing the coverslip from the flask and staining and mounting it.

Examining microscopically a random sample of 500 macrophages and determining their pigment content.

Obtaining the phagocytic index of the culture, i.e., pigment clumps per macrophage.

Variations of this procedure, necessary to answer specific problems, will be indicated in the text.

D. The in vitro opsonic test applied to *P. gallinaceum*

1. The standard opsonic test with *P. gallinaceum* and immune or normal test serum: experiment 4.—

TABLE 5.—Exp. 4. Phagocytic indices in standard opsonic cultures with *P. gallinaceum* and test serums from normal chickens and from chickens with patent or subpatent infections.

Test serum	Day after inoculation when serum was collected	Phagocytic indices							Mean
		0.01-0.20	0.21-0.30	0.31-0.40	0.41-0.50	0.51-0.60	0.61-0.70	0.71-0.80	
Acute rise	0- 13	1							0.11 ± 0.01
	14- 50			2	2				
	51-100		2		3	2		1	
	101-150						1		
Subpatent (Immune)	151-200			1	1	2			
	201-250								
	251-300			1				1	
	301-350				1				
	351-400								
	Total of subpatent indices		2	4	7	4	1	2	0.45 ± 0.01
Normal			5	6	5	2	2		0.40 ± 0.0

Serum samples were collected from 20 chickens with infections of *P. gallinaceum* which had been latent for from 3 to 380 days and from 1 chicken during the acute initial infection. The birds ranged in size from 400 to 1,900 g. Control serum samples were collected at the same time from 20 normal uninfected chickens, ranging in size from 200 to 1,800 g. The 41 samples thus collected were tested in vitro by the technic previously described, and their phagocytic indices are listed in Table 5.

From these data, it may be seen that the phagocytic indices of the normal serums ranged from 0.21 to 0.67 mean pigment clumps per macrophage and their mean was 0.40 ± 0.01 . The phagocytic indices of the immune serums fell approximately within the same range, although 2 of them were higher. Their

mean was 0.45 ± 0.01 . The phagocytic index of the serum drawn during the acute initial infection with *P. gallinaceum* was 0.11 ± 0.01 . This figure was lower than any of the indices of normal serums and was significantly lower than their mean.

The results of experiment 4 indicate that no more circulating opsonin occurred in the serums of birds with latent infections of *P. gallinaceum* than in normal birds, and a smaller amount than in normal birds occurred in the blood of a chicken during an acute initial infection with *P. gallinaceum*.

2. The opsonic test (macrophages grown in plasma) with *P. gallinaceum* in contact 5 minutes to 24 hours with hyperimmune or normal test serum: experiment 5.—The technic used in this experiment differed from that previously described in that plasma rather than serum was used throughout and the cultures were fixed at varying times.

Chicken blood buffy coat macrophages, prepared in the standard manner, were grown in plasma containing an excess of heparin—1 ml of 1:500 heparin (Connaught Laboratories) made up in physiological salt solution for every 9 ml of blood drawn. The buffy coat from the heparinized blood had a tendency to form a "pancake" on the surface of the erythrocytes and was, therefore, unsuitable for use in the inoculum. Plasma was also used instead of serum in the opsonic test and was drawn from a chicken 11 days after the last of 3 superinfections given during the course of 1 month to hyperimmunize the animal. Normal

chicken plasma drawn at the same time served as a control.

The antigen consisted of washed blood containing 101 parasites in 100 red cells from an acute initial infection with *P. gallinaceum*.

Macrophage cultures to test the normal and hyperimmunized plasmas were rinsed and fixed after the following periods of incubation in the presence of the parasitized blood suspensions: 5, 15 and 30 minutes, and 1, 2, 4, 8, 12, 18 and 24 hours. A similar set of cultures was concurrently exposed to suspensions of normal washed red blood cells in the same plasma samples.

Table 6 presents the phagocytic indices of the 2 series of cultures in contact with the parasitized blood. Whereas in both of the series the amount of

in both of the 24 hour cultures is not understood since the macrophages had probably not begun to digest pigment within this comparatively short period.

Only 2 of the 3 adhesion phenomena, previously described, i.e., adhesion and rosette formation, were seen in the hyperimmune plasma cultures. The third reaction, agglutination, also probably took place, although no data were collected, since all 3 reactions regularly occurred in the presence of hyperimmune serum in other experiments.

In cultures exposed to infected or normal red cells in hyperimmune plasma,

TABLE 6.—Exp. 5. *Phagocytic indices in opsonic cultures (macrophages grown in plasma) with P. gallinaceum in contact with hyperimmune or normal test plasma from 5 minutes to 24 hours.*

Period of exposure of macrophages to parasitized cells in test plasma	Test plasma				t*
	Hyperimmune		Normal		
	Culture number	Phagocytic index	Culture number	Phagocytic index	
5 minutes	74	0.01±0.003	84	0.0	
15 minutes	75	0.05±0.01	85	0.002±0.002	4.08
30 minutes	76	0.07±0.01	86	0.01±0.004	4.44
1 hour	77	0.20±0.02	87	0.04±0.01	6.15
2 hours	78	0.43±0.04	88	0.09±0.02	7.52
4 hours	79	0.86±0.06	89	0.21±0.03	9.85
8 hours	80	1.66±0.08	90	0.57±0.04	11.54
12 hours	81	2.44±0.10	91	0.91±0.06	13.58
18 hours	82	3.96±0.13	92	1.67±0.09	14.69
24 hours	83	3.23±0.12	93	1.25±0.12	11.72

* t: difference between normal index and hyperimmune index divided by the standard error of the difference.

phagocytosis increased from the beginning through the 18th hour of the experiment, phagocytosis began earlier and was more intense in the cultures exposed to hyperimmune plasma than in normal plasma. Thus, by the 8th hour of incubation, the macrophages exposed to parasitized blood in hyperimmune plasma had taken up as much parasitic material as the macrophages in normal plasma ingested in 18 hours. The high values of "t" in Table 6 show that the hyperimmune serum cultures were throughout significantly more phagocytic than their companion controls. Furthermore, the degree of significance of difference increased progressively until the 18th hour of incubation. The reason for the fall in phagocytic index

adhering red cells were definitely discernible after 15 minutes of incubation, were most numerous after 0.5, 1 or 2 hours of incubation, and had practically disappeared after 4 hours of incubation by which time they had probably been ingested by the macrophages. No adhering red cells were seen in cultures exposed to normal plasma.

Rosettes were marked after 1 hour in hyperimmune plasma, but they, also, tended to disappear with further incubation. They were more numerous in cultures containing parasitized blood than in those containing normal blood.

The nonspecific nature of the adhesion phenomena was demonstrated by the fact that adhering red cells were found in the cultures containing hyper-

immune plasma with a suspension of normal red cells as well as in those containing parasitized red cells.

To sum up experiment 5, the presence of opsonin in the plasma of chickens hyperimmunized against *P. gallinaceum* has been detected by the following reactions:

1. Phagocytosis of both normal and parasitized red cells was more rapid and more intense in hyperimmune plasma than in normal plasma as measured by the phagocytic index. In addition, the macrophages in hyperimmune plasma were stimulated to ingest parasites and parasitized red cells, whereas those in normal plasma tended to ingest free malarial pigment.

2. Erythrocytes, both normal and parasitized, adhered to the flask base, and erythrocyte rosettes formed around macrophages in hyperimmune plasma and not in the normal-plasma controls.

3. *The absorption of phagocytic and adhesive factors from hyperimmune serum with normal or P. gallinaceum-parasitized red cells: experiment 6.*—This experiment was undertaken to determine whether the absorption of the agglutinin found in serum from chickens hyperimmunized against *P. gallinaceum* would affect the phagocytic index of such serum and whether the agglutinin could be absorbed by normal as well as by parasitized red cells.

A sample of normal chicken serum was divided into 3 parts. One 2.5 ml portion was incubated for 2 hours together with the washed normal chicken erythrocytes from 5 ml of whole blood. A second portion was incubated with an equivalent quantity of washed blood cells heavily parasitized with *P. gallinaceum* (112 parasites in 100 red blood cells). A third sample was incubated without any cells. All samples were shaken frequently during the incubation period.

Three parallel portions of hyperimmune serum were identically treated. This serum had been collected 7 months before the experiment from a chicken which had been 7 times superinfected with *P. gallinaceum*.

Since the absorbed hyperimmune serum samples still agglutinated *P. gallinaceum*-infected blood, each sample was reincubated for 3.75 hours with the same type of cell with which it had already been treated. After the second incubation, the antigallinaceum agglutinin was completely removed from the hyperimmune serum by both normal and parasitized erythrocytes. These various serums were then diluted 1.5 times with modified Tyrode's solution and tested by 1 hour's contact with the antigen in cultures 94 through 99.

Agglutination of the suspended cells was seen only in culture 94, which contained the unabsorbed hyperimmune serum.

Since, in addition to phagocytosis, the adhesion phenomena were to be studied, the period of exposure to the antigen was reduced to 1 hour. As a result of the short period of exposure, the phagocytic indices based on pigment clumps were low (although the difference between indices of unabsorbed hyperimmune serum, culture 94, and unabsorbed normal serum, culture 97, was statistically significant) and an additional measure of phagocytic activity was included, viz., erythrophagocytosis. The fact that variations in percentages of macrophages exhibiting +++ erythrophagocytosis of both normal and parasitized red cells closely followed variations in phagocytic indices based on pigment clumps supports the validity of this additional type of analysis.

The phagocytic indices and the percentage of +++ erythrophagocytosis for these cultures are given in Table 7. Macrophages were assigned to this category if they had taken up so many normal or parasitized red cells that their own cytoplasm could not be seen between the ingested cells.

Although the phagocytic indices were low in all cultures due to the short period of exposure, the index was as low in culture 95, which contained hyperimmune serum absorbed with normal blood cells, as in cultures 97 through 99 which contained absorbed or untreated normal serum. The amount of erythrophagocytosis was also similar in these cultures. Absorption with para-

sitized red cells only partially removed the opsonic factor from hyperimmune serum (cf. culture 96 with 94). There were also traces of rosette formation and of adhering red cells in culture 96. This finding bears out the observation in experiment 6 that the stimulated erythrophagocytosis in hyperimmune serum was at least in part nonspecific in that normal as well as parasitized cells were ingested.

The results of this experiment support the identity of the agglutinating, rosette-forming and opsonizing antibodies because the complete removal of

Buffy coat macrophage cultures were prepared in the standard manner from the leucocytes of normal chickens.

Two sets of 4 chickens were used as donors of parasitized red cells during their initial infections with *P. gallinaceum*, when they had the following numbers of parasites per 100 red cells: 60, 66 and 76 on 3 successive days during the acute rise; 102, 182 and 198 at the peak of the infection; 118 during the crisis; and 58 on the day after the crisis. From each of these antigen samples, 4 dilutions were used for 4 cultures, and the numbers of parasites and red cells introduced into each flask were calculated from hemocytometer counts and tetrachrome-stained smears. Since the chickens did not contain the same number of red cells or of parasites per cmm whole blood, the absolute number of red cells or parasites varied

TABLE 7.—Exp. 6. *Phagocytic indices and extent of erythrophagocytosis in standard opsonic cultures with P. gallinaceum after absorption of the hyperimmune or normal test serum with normal or parasitized red cells.*

Type of red cell with which serum was absorbed	Test serum					
	Hyperimmune			Normal		
	Culture number	Phagocytic index	% of macrophages with +++ erythrophagocytosis*	Culture number	Phagocytic index	% of macrophages with +++ erythrophagocytosis*
None	94	0.10	88	97	0.01	0
Normal	95	0.01	1	98	0.01	1
<i>P. gallinaceum</i> infected	96	0.03	51	99	0.01	0

* Erythrophagocytosis involved both normal and parasitized red cells.

the agglutinin was accompanied by the removal of all the other factors, whereas the partial removal of the adhesion factor (agglutination suppressed but rosette-formation and adhering red cells still demonstrable) only partially removed the factor responsible for stimulated phagocytosis.

4. *The opsonic test using P. gallinaceum (from various stages of initial infections and in different dilutions in the cultures) and normal test serum: experiment 7.*—The object of this experiment was to determine whether circulating parasites are opsonized in vivo during infection with *P. gallinaceum*. A further aim was to see whether varying the number of parasites in a culture would affect the phagocytic index.

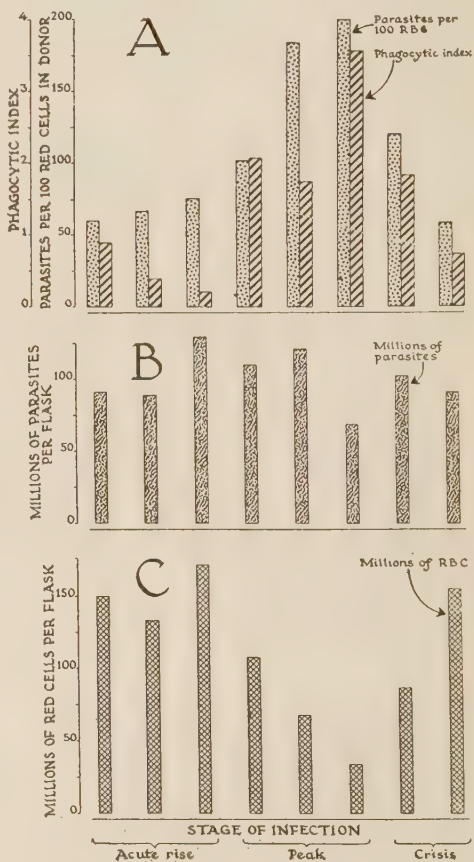
in the 8 blood samples. For example in the 31 cultures (1 culture broke), the parasites varied from 22 to 271 million and the red cells from 29 to 228 million (see Tables 8 and 9). In each set, however, there was one culture that contained approximately 100 million parasites and another culture (or even the same one) that contained approximately 100 million red cells.

Erythrocytes adhering to the cover-slip and rosette formation were not observed in one set of cultures, but were observed in the other set although the chicken serum in both sets of cultures was normal. This puzzling occurrence of a phenomenon, interpreted in other experiments as due to the presence of antibody, might be explained if we assume the presence of incompatible blood types in chickens. Aside from the foregoing difference, the results in the 2

sets of cultures corroborated each other, as may be seen in the following description of the results.

When cultures containing as nearly as possible 100 million parasites were chosen from each of the foregoing 8 groups representing 8 antigen samples (See Tables 8 and 9) and were arranged in order with respect to the varying stages of the acute initial infection at which the blood was drawn, their phagocytic indices varied directly with the stage of the infection and not with the number of parasites introduced into the cultures. Thus, the highest index was coincident with the peak of the infection, as may be seen in Graph 1. The initial fall in phagocytic index during the early acute rise of the infection is probably a result of the binding of normal opsonin by the accumulating antigen before immune antibody was formed. Furthermore, when the phagocytic indices were similarly plotted for those cultures (1 out of each group of 4), which contained approximately 100 million red cells instead of 100 million parasites, the phagocytic index again reflected the stage of the infection and not the number of red cells introduced into the cultures, as may be seen in Graph 2. In other words, the phagocytic index of cultures containing parasites from different stages of the infection increased as the crisis of the infection was reached and decreased after the crisis when either the number of parasites or of red cells per culture was held constant. The apparent inverse relationship between the phagocytic index and the number of red cells per culture in Graph 1, and the apparent direct relationship between the phagocytic index and the number of parasites per culture in Graph 2, are incidental and not essential, since when either of these factors in turn is kept constant the phagocytic indices are not thereby appreciably affected.

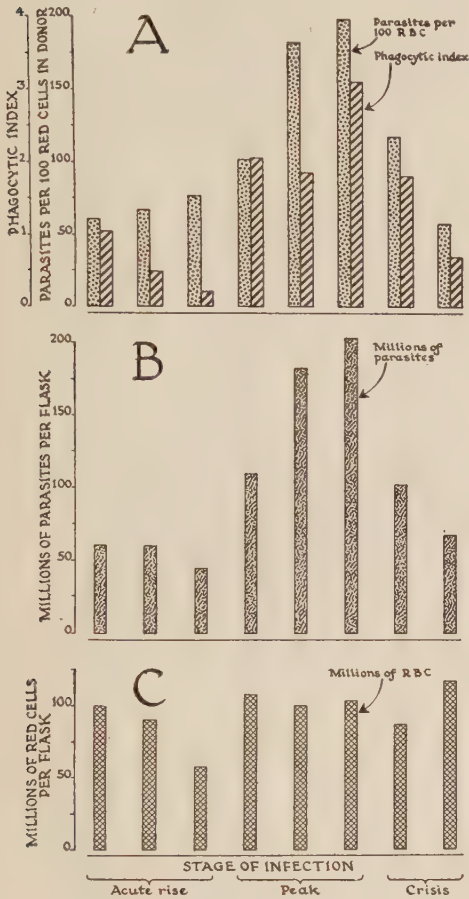
Since normal serum was used in this experiment, the serum itself could not have been responsible for the variations in phagocytic index. Evidently the parasitized red cells at the peak and crisis of the infection were so altered (i.e., opsonized) *in vivo* as to be more readily



GRAPH 1.—A, Phagocytic index and number of parasites per 100 red cells in the donor chicken; B, number of parasites per flask; and C, number of red cells per flask in 8 macrophage cultures containing normal serum and *P. gallinaceum*-infected red cells collected at 8 progressive points during the initial infections of 8 chickens. The cultures were incubated 4 hours with parasites and 20 hours without parasites. Each culture contained approximately 100 million parasites (B), whereas the red cells per culture decreased progressively and then increased (C).

Note that the phagocytic index varied as did the number of parasites in the donor chickens and not as did the number of parasites per culture.

taken up by the culture macrophages than were similar parasitized red cells from other stages of the infection. Also the bond between the parasitized cells and the absorbed opsonin was stable because it was not disturbed by vigorous washing.



GRAPH 2.—Same as in Graph 1. In this graph the parasites per culture progressively increased then decreased (B), whereas each culture contained approximately 100 million red cells (C).

Note that the phagocytic index varied directly as did the number of parasites in the donor chickens and not as did the number of red cells per culture.

The phagocytic index was not appreciably affected by the dilution of antigen collected during the acute rise of initial infections, but, when blood from

the peak of an infection was used, the more dilute the antigen, the higher the phagocytic index, within the limits of the experiment (Tables 8 and 9). The same pattern was followed when blood was drawn during the crisis, i.e., 1 day after the peak in number count had been reached (see Table 8, cultures 112 through 115), but was not effective as the crisis progressed into the developed infection (Table 9, cultures 128 through 131). Thus, the dilution factor, whatever its nature, occurred only at the peak and crisis of the infection.

The observed inverse relationship between the phagocytic index and the amount of antigen introduced into a culture, when the antigen was obtained at the peak or during the early crisis of an infection, is not clearly understood. Crowding could not have been responsible since such variations did not occur if the antigen was obtained during the acute rise of an infection. It may be that the optimum concentration of some nonspecific factor in normal serum led to the observed increase in the phagocytosis of opsonized cells. The fact that the phagocytic indices of cultures 126 and 127 (see Table 9) were so close together suggests that further dilution might not have raised the index beyond those already observed. In other words, the optimum concentration of the postulated nonspecific factor may have been reached.

Experiment 7 may be summarized as follows:

(1) Parasitized red cells drawn from chickens at the peak and crisis of initial infections with *P. gallinaceum* were opsonized in vivo. Those drawn during the acute rise of an infection or after the crisis were opsonized either to a limited extent or not at all in vivo.

(2) Varying the number of parasitized red cells from the acute rise or after the crisis of initial infections with *P. gal-*

linaceum did not alter the phagocytic index in vitro, but decreasing the number of parasitized red cells at the peak or crisis of the infection increased the phagocytic index.

chicken at various stages of its infection, and when the antigen was tested in hyperimmune as well as in normal serum.

It was necessary to prepare standard cultures

TABLE 8.—Exp. 7. *Phagocytic indices in opsonic cultures with P. gallinaceum in normal test serum. In these cultures the parasitized blood varied with respect to the stage of the infection in the donor bird and the number of parasites and red cells per culture.*

Parasitized blood collected from chicken		Culture number	Parasites per culture (millions)	Erythrocytes per culture (millions)	Phagocytic index
Stage of infection	Parasites per 100 red cells				
Acute rise	60	100	120	200	1.25
		101	90	150	0.87
		102	60	100	1.04
		103	30	50	1.48
Peak	182	104	242	133	1.13
		105	182	100	1.84
		106	121	67	1.72
		107	61	34	4.74
Peak	198	108	271	137	2.17
		109	203	103	3.12
		110	136	69	2.91
		111	68	34	3.52
Crisis	118	112	136	115	1.27
		113	102	86	1.80
		114	68	58	1.42
		115	34	29	2.69

TABLE 9.—Exp. 7. *Phagocytic indices in opsonic cultures with P. gallinaceum in normal test serum. In these cultures the parasitized blood varied with respect to the stage of the infection in the donor bird and the number of parasites and red cells per culture.*

Parasitized blood collected from chicken		Culture number	Parasites per culture (millions)	Erythrocytes per culture (millions)	Phagocytic index
Stage of infection	Parasites per 100 red cells				
Acute rise	66	116	118	179	0.42
		117	88	133	0.39
		118	59	89	0.46
		119	29	44	0.43
Acute rise	76	120	173	228	0.15
		121	129	170	0.20
		123	43	57	0.20
Peak	102	124	145	142	1.16
		125	109	107	2.07
		126	73	72	3.09
		127	36	35	3.14
Post crisis	58	128	90	155	0.71
		129	67	116	0.70
		130	45	78	0.45
		131	22	38	0.42

5. *The opsonic test with P. gallinaceum (taken at 18 hour intervals from 1 chicken during an initial infection) and with hyperimmune or normal test serum (1:1): experiment 8.*—In experiment 7, the antigen was collected from 8 chickens. The purpose of experiment 8 was to determine whether the results of experiment 7 could be corroborated when the antigen was drawn from the same

in 4 series at 18 hour intervals. The antigen consisted of 4 blood samples, drawn at 18-hour intervals from the same chicken, beginning at a time when 1 set of the macrophage cultures was 24 hours old. The parasite number counts per 100 red cells were as follows:

- 1—64 parasites during the acute rise of the infection.
- 2—117 parasites during the acute rise of the infection.
- 3—200 parasites at the peak.
- 4—122 parasites at the crisis.

To 24-hour old cultures were added approximately 50 million of the particular red cells to be tested, along with normal serum or hyperimmune serum, diluted 1:1 in modified Tyrode's solution. The hyperimmune serum was collected 12 days after a chicken had been superinfected for the seventh time with *P. gallinaceum*. A 1:1 dilution of serum was made in an attempt to limit the amount of agglutination found when hyperimmune serum was present in cultures.

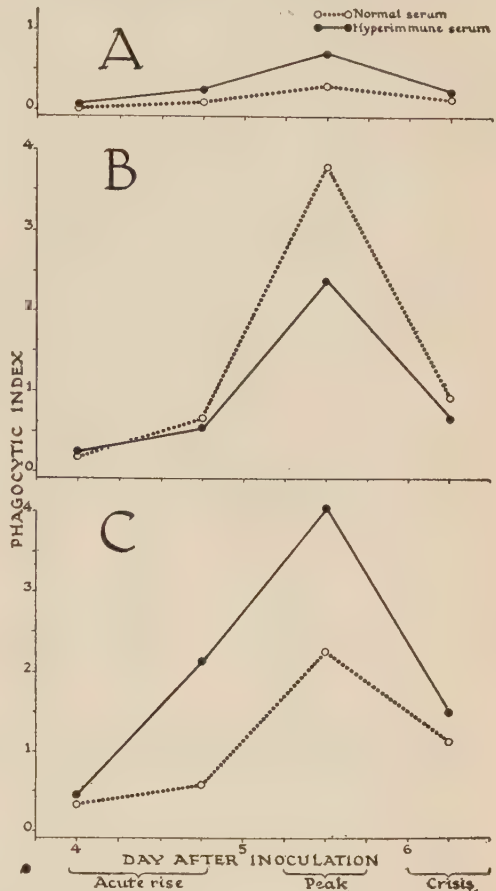
Cultures from each series were washed and fixed after the following periods of incubation with the antigen inoculum: 1 hour, 4 hours and 4 hours with subsequent washing and reincubation for 20 hours in normal serum.

Throughout this experiment, the parasitized blood cells agglutinated in all of the cultures containing hyperimmune serum and in none of the cultures containing normal serum. Rosette formation and the adhering of normal and parasitized red cells to the coverslip were seen in all cultures containing hyperimmune serum except in those containing red cells from the peak of the infection after 4 hours contact and reincubation for 20 hours in normal serum. This finding is probably due to the fact that the parasitized cells in these cultures were more vigorously phagocytosed than were cells from other stages of the infection as indicated by higher phagocytic indices.

The phagocytic indices of the cultures in this experiment are presented in Graph 3 and corroborate the findings with respect to normal serum in experiment 7. They also showed the same trends in hyperimmune serum. It might be argued that the pattern of the phagocytic indices at various stages of the infection simply reflected the number of parasites per flask and not the previous history of in vivo opsonization of the parasitized cells, but this view is untenable because in experiment 7, when parasitized blood was drawn from the peak of an infection, the fewer the parasitized red cells introduced into a culture, the higher the phagocytic index.

Thus, had the number of parasites been constant, the phagocytic index from the peak of the infection would have been even higher than the observed index.

The indices were in general higher in



GRAPH 3.—Phagocytic index in macrophage cultures containing normal or hyperimmune serum and *P. gallinaceum*-infected red cells collected 4 times at 18-hour intervals during an initial infection of the same chicken. The cultures were incubated with parasites for 1 hour (A), for 4 hours (B) or for 4 hours followed by 20 hours without antigen (C). The first 3 cultures contained progressively more parasites and the last one fewer parasites, whereas all cultures contained approximately 50 million erythrocytes.

Note that the phagocytic index varied as in Graphs 1 and 2, i.e., directly as did the number of parasites in the donor chicken and that, in general, it was higher in the cultures containing hyperimmune serum than in those containing normal serum.

the cultures containing hyperimmune test serum than in those containing normal test serum. In the last 3 of the 4 sets of cultures exposed to the antigen in serum for 4 hours and fixed immediately, however, the indices of the cultures exposed to hyperimmune serum were lower than those of the normal controls. In the opinion of the author, this finding can be ascribed to the interference with phagocytosis by agglutination of the antigen. Cells taken during the earlier part of an infection were less opsonized

in hyperimmune serum were able to feed upon the adhering red cells during the reincubation period while those in normal control serum had no adhering red cells to devour. The fact that the indices of the hyperimmune serum cultures fixed after a single hour of exposure were higher than the normal controls does not necessarily contradict the suggestion that agglutination interfered with phagocytosis. It was seen in a previous experiment (experiment 5) that the presence of opsonin results not

TABLE 10.—Exp. 8. *Macrophages which ingest pigment alone or pigmented parasites and degrees of erythrophagocytosis in opsonic cultures of P. gallinaceum after 1 hour with hyperimmune or normal test serum. In these cultures the parasitized blood varied with respect to the stage of the infection in the donor bird.*

Test serum	Blood collected from chicken		Culture number	% of macrophages		% of macrophages with erythrophagocytosis*		
	Stage of infection	Parasites per 100 red cells		With pigment	With parasites	0**	±**	+++**
Hyperimmune	Acute rise	64	132	44	58	2	25	73
		117	133	16	84	18	9	73
	Peak	200	134	8	92	2	8	90
	Crisis	122	135	20	80	7	6	87
Normal	Acute rise	64	136	100	0	45	55	0
		117	137	80	20	59	41	0
	Peak	200	138	50	50	45	54	1
	Crisis	122	139	38	62	50	48	2

* Erythrophagocytosis involved both normal and parasitized red cells.
** 0 = no erythrophagocytosis observed; ± = slight erythrophagocytosis observed; +++ = extensive erythrophagocytosis observed.

in vivo and therefore would be less agglutinated by the hyperimmune serum than the cells taken later in the infection. As shown by the cultures which were were reincubated for an additional 20 hours in normal serum (Graph 3, C), the macrophages of the hyperimmune serum cultures in time more than made up for their temporary lag in phagocytic activity. Since washing the cultures removed neither the sticky red cells adhering to the coverslips nor the rosettes of normal and parasitized erythrocytes surrounding the macrophages, and since such adhering cells were peculiar to cultures containing hyperimmune serum, the macrophages

only in a more rapid rate of phagocytosis but also in an earlier initiation of this process. Consequently, the macrophages in normal serum could have lagged behind those exposed to hyperimmune serum during the first hour.
In addition to phagocytic indices, an analysis was made of the tendency of macrophages in hyperimmune serum to take up recognizable free parasites and parasites in erythrocytes rather than to scavenge free pigment, as do macrophages in normal serum. In each of the cultures exposed for 1 hour to the test inoculums, 100 macrophages containing pigment were chosen at random and tabulated for the presence or ab-

sence of recognizable parasite cytoplasm around ingested pigment.

The results of this survey are summarized in Table 10. The macrophages in the category "with pigment" contained only pigment. Those in the category "with parasites" contained the cytoplasm of at least 1 parasite although free pigment may also have been found. The ingested whole parasites were either free or in erythrocytes. These data reflect the influence of both in vivo and in vitro opsonization in stimulating the phagocytosis of parasites and parasitized red cells. In control culture 136 in which there had been

These trends were also reflected by erythrophagocytosis of both normal and parasitized cells, as may be seen in Table 10. The difference in the degree of erythrophagocytosis between cultures exposed to parasitized red cells in normal and hyperimmune serums was marked and was associated not only with the presence of hyperimmune serum in the culture, but also with the opsonization of the parasitized blood in vivo. Thus, the greatest amount of erythrophagocytosis was found in culture 134.

Since macrophages exposed to opsonized blood differed from the normal

TABLE 11.—Exp. 9. *Phagocytic indices in standard opsonic cultures with P. lophurae and immune or normal test serum.*

Test serum	Day after inoculation when serum was collected	Phagocytic indices				Mean
		0-0.10	0.11-0.20	0.21-0.30	0.31-0.40	
Subpatent (immune)	0-50		3			
	51-100	1	10	2		
	101-150		1			
	201-250	1	2			
	Total	2	16	2		0.17 ± 0.004
Normal		3	15	1	1	0.15 ± 0.003

least in vivo opsonization of the parasitized blood and in which no serum opsonin was present, only free pigment was phagocytosed. Then, as the parasitized red cells were obtained progressively later in the infection and thus were increasingly opsonized in vivo, more recognizable parasites were taken up and fewer macrophages ingested free pigment alone. On the other hand, in culture 132, in which there was the same amount of in vivo opsonization of the antigen as in culture 136 but in which hyperimmune serum was present, more than half of the macrophages contained recognizable parasites and/or parasitized red cells. Then, as the in vivo opsonization became progressively greater in cultures 133 and 134, stimulated phagocytosis increased.

controls not only in their more vigorous phagocytosis of parasitized cells, but of normal erythrocytes as well, the latter, as well as the parasitized cells, were opsonized.

The results of experiment 8 may be summarized as follows: (1) The opsonization in vivo of parasitized red cells from the peak of an infection with *P. gallinaceum*, as demonstrated in experiment 7, has been corroborated, using as antigen parasitized blood from the same chicken at various stages during its initial infection. (2) The presence in hyperimmune serum of an opsonin against normal and *P. gallinaceum*-infected blood cells has been demonstrated with respect to the phagocytic index, scavenging vs. stimulated phagocytosis, and erythrophagocytosis.

E. The in vitro opsonic test applied to P. lophurae

1. *The standard opsonic test with P. lophurae and immune or normal test serum: experiment 9.*—The object of this experiment was to compare the in vitro phagocytosis of *P. lophurae*-infected red cells in normal serums and in serums from chickens with latent *P. lophurae*

chicken 5 days after the last of 4 superinfections with *P. lophurae*.

The antigen was collected during the early acute rise of an infection with *P. lophurae* (51 parasites in 100 red cells).

Cultures containing hyperimmune or normal serum were fixed after 0.5, 1, 3, 5.5, 9.25, 12 and 24 hours of incubation in the presence of the parasitized blood cell suspensions.

Rosette formation and adhering red cells were found in the cultures exposed

TABLE 12.—Exp. 10. *Phagocytic indices with P. lophurae in contact with hyperimmune or normal test serum from 0.5 to 24 hours.*

Hours of exposure of macrophages to parasitized cells in test serum	Test serum				t*
	Hyperimmune		Normal		
	Culture number	Phagocytic index	Culture number	Phagocytic index	
0.5	180	0.13±0.02	187	0.08±0.01	2.25
1.0	181	0.29±0.03	188	0.22±0.03	1.75
3.0	182	0.44±0.04	189	0.43±0.04	2.00
5.5	183	0.55±0.04	190	0.40±0.04	2.60
9.25	184	1.29±0.07	191	0.71±0.06	6.56
12.0	185	2.14±0.08	192	0.73±0.06	14.25
24.0	186	7.49±0.18	193	3.74±0.12	16.70

* t: difference between the normal index and the hyperimmune index divided by the standard error of the difference.

infections. The immune serum donors ranged in size from 450 to 1,300 g and the normal serum donors ranged from 200 to 1,800 g. The normal serums were the same ones as used in experiment 4.

The technic was standard and the test antigen was collected during the acute rise of an initial infection with *P. lophurae* (44 parasites in 100 red cells).

No agglutination of the supernatant cells was seen in any of the cultures.

As may be seen in Table 11, the phagocytic indices of the cultures exposed to immune serums fell within the range of the normal control indices. Therefore no opsonin was found in the immune serums.

2. *The opsonic test with P. lophurae in contact 0.5 to 24 hours with hyperimmune or normal test serum: experiment 10.*—Since there was no demonstrable opsonin against *P. lophurae* in latent serum, cultures containing hyperimmune as compared to normal serum were tested.

The hyperimmune serum was taken from a

to hyperimmune serum, but not in those containing normal serum. As in the *P. gallinaceum* material discussed elsewhere, rosettes were most marked in the cultures incubated for short periods (0.5 hr. and 1 hr.), were clearly seen in those incubated through 5.5 hours, but were seen only as traces after longer periods of incubation. Adhering red cells were seen in the entire hyperimmune serum series except in the culture exposed for 12 hours. No data are available as to whether the cells agglutinated in the hyperimmune serum, as the supernatant fluids had been discarded by the time the adhesion phenomena began to be studied.

The phagocytic indices of the cultures in this experiment are listed in Table 12. Inspection of the values of "t" shows that differences between hyperimmune and normal serum cultures at the ninth hour of incubation and thereafter were significant, and that although no statistically significant dif-

ferences occurred before this time, the hyperimmune series were throughout somewhat more phagocytic than the normal controls.

This experiment demonstrates the presence of a low-titer antibody against *P. lophurae*-infected blood in hyperimmune serum.

3. *The standard opsonic test with P. lophurae and hyperimmune or normal test serums: experiment 11.*—The results obtained in experiment 10 suggested testing other hyperimmune serums against *P. lophurae*.

The hyperimmune serums were obtained from 8 adult cockerels 16 days after the last of 4 superinfections given over a period of 3 months. Normal serums were collected from 10 young cockerels, from 1 full grown cock (culture 212) and from the pooled serum of several full-grown cocks (culture 213). All test serums were collected 4 to 6 days before culturing.

The antigen was a sample of blood collected during the acute rise of an initial infection with *P. lophurae* (50 parasites in 100 red cells).

The macrophages were prepared by the standard technic with exposure to antigen and serum for 4 hours and reincubation for 20 hours in normal chicken serum. Macrophages exposed to normal serum are shown in Fig. 8 and 9 in Plate 1; and to hyperimmune serum in Fig. 10 and 11.

Agglutination of the free cells occurred in all of the cultures containing hyperimmune serum, but in none of those containing normal serum. Also adhering red cells and rosettes were seen in 5 of the 8 cultures exposed to hyperimmune serum even after 20 hours of reincubation. Of the 3 cultures negative for adhesion and rosettes, 2 (cultures 196 and 201) had "normal" phagocytic indices and another (culture 198) contained serum from a chicken which was moribund when bled for serum and unaccountably died 2 days later.

As may be seen from Table 13, 5 of 8 cultures containing hyperimmune serum had phagocytic indices which were significantly higher than the indices of those containing normal serums. That

the opsonin was present in considerable quantities in the 5 cultures (194, 195, 197, 199 and 200) is attested by the large values of "t."

Although no circulating opsonin was demonstrated in cultures 196, 198 and 201, all of the hyperimmunized birds were definitely resistant to superinfection—a fact which proves that their immune mechanisms were actively functioning. If large numbers of parasitized cells introduced intravenously bind free circulating antibody and time is needed to produce more antibody and to deliver

TABLE 13.—Exp. 11. *Phagocytic indices in standard opsonic cultures with P. lophurae and hyperimmune or normal test serum.*

Test serum				
Hyperimmune			Normal	
Culture number	Phagocytic index	t*	Culture number	Phagocytic index
194	2.05 ± 0.09	9.5	202	1.10
195	9.13 ± 0.17	47.9	203	1.12
196	1.27 ± 0.08	1.0	204	0.71
197	2.78 ± 0.09	18.0	205	1.21
198	0.35		206	0.95
199	3.17 ± 0.13	13.8	207	1.33
200	4.61 ± 0.22	15.8	208	0.90
201	1.17 ± 0.05	0.3	209	1.26
			210	1.44
			211	1.22
			212	1.21
			213	1.79
			Mean	1.19 ± 0.08

* t: difference between normal index computed from all normal serums and hyperimmune index divided by the standard error of the difference.

it to the circulating blood, individual variations may exist in the rate of recovery of different birds from the superinfecting dose. If it be argued that antibodies are locally produced by the macrophages in closest contact with the antigen, i.e., in malaria by the macrophages of the blood-filtering organs, the spleen, liver and bone marrow, and that there is an overflow of antibody into the circulation only after the macrophages produce a certain threshold quantity of antibody, individual variations may exist in the threshold concentration beyond which excess opsonin may circulate or in the speed of attaining this level. Furthermore, massive agglutina-

tion of the antigen, which has already been discussed as another possible explanation of the low phagocytic indices in some cultures exposed to hyperimmune serum, may have masked the presence of opsonin in these cultures.

4. *The standard opsonic test with P. lophurae and hyperimmune or normal test serum after an additional superinfection of the hyperimmune chickens in experiment 11: experiment 12.*—The ob-

tion and reincubation periods of 4 hours and 20 hours, respectively.

Agglutination of the supernatant cell suspensions occurred in all of the cultures containing hyperimmune serums and in none of those containing normal serum.

Examination of Table 14 indicates that all 7 phagocytic indices of the cultures containing hyperimmune serum were significantly higher than those of

TABLE 14.— Exp. 12. *Phagocytic indices in standard opsonic cultures with P. lophurae and hyperimmune or normal test serum.*

Culture in Table 13 using same bird as serum donor	Test serum						
	Hyperimmune			Normal			
	Culture number	Phagocytic index	t*	Chicken number	Days stored at refrigerator temp.	Culture number	Phagocytic index
194	214	0.65 ± 0.04	4.6	Normal pooled ♂ serum	71	221	0.55
195	215	3.29 ± 0.08	34.6	Normal pooled ♂ serum	55	222	0.43
196	216	0.73 ± 0.05	5.4	I	48	223	0.56
197	217	0.78 ± 0.03	10.3	II	48	224	0.32
199	218	0.71 ± 0.03	7.1	III	48	225	0.41
200	219	0.84 ± 0.04	9.3	IV	48	226	0.45
201	220	1.02 ± 0.05	11.9	V	48	227	0.39
				VI	48	228	0.39
				VII	48	229	0.42
				VIII	48	230	0.51
				IX	48	231	0.42
				X	48	232	0.43
				3095	48	233	0.46
				3095	0	234	0.46
						Mean	0.46 ± 0.008

* See footnote of Table 13.

ject of this experiment was to determine how an additional superinfection with *P. lophurae* and a shorter time interval between superinfection and bleeding for serum would affect the phagocytic index. Also, the effect of storing normal serums at refrigerator temperature was studied.

Whereas the hyperimmune serums in experiment 11 were drawn 16 days after the 4th superinfection, those in the present experiment were collected on the 7th day after the 5th superinfection of the same chicken and were stored at 6 C for 14 days before use. Normal test serums were stored at 6 C for from 0 to 71 days. Those marked I-X in Table 14 were identical with the normal serums used in experiment 11.

The antigen was collected from a chicken during the acute rise of its initial infection with *P. lophurae* (52 parasites in 100 red blood cells).

The cultures were given the standard incuba-

tion of the antigen, which has already been discussed as another possible explanation of the low phagocytic indices in some cultures exposed to hyperimmune serum, may have masked the presence of opsonin in these cultures. The additional superinfection and the shorter interval between superinfecting and bleeding may both have contributed to this effect. The observed differences between the respective indices, however, indicates that the opsonic titer of some of the hyperimmune serums evidently fell after the additional superinfection. This drop may have been due to the fact that some chickens had not as yet fully compensated for the large dose of antigen received when superinfected.

The considerable difference between the phagocytic index of the cultures exposed to normal serum in Table 14 (Mean = 0.46 ± 0.008) and in Table 13 (Mean = 1.19 ± 0.08) indicates clearly

that comparisons between sets of cultures cannot be legitimately made. In other words, conditions are similar with respect to components and technique in cultures made at a given time, but vary in cultures made at different times (cf. also exp. 2, 6, 7 and 8).

5. *The opsonic test with P. lophurae in contact 1 hour with hyperimmune or normal test serum: experiment 13.*—In this experiment, the adhesion phenomena

slip and their persistent radial arrangement in rosettes around the macrophages were all observed in the cultures containing hyperimmune serum and not in the control cultures.

Other essential data of this experiment are summarized in Table 15. The difference between the pooled phagocytic indices of the normal as contrasted with the hyperimmune serum cultures was slight, but statistically significant,

TABLE 15.—Exp. 13. *Macrophages which ingested pigment alone or pigmented parasites and degrees of erythrophagocytosis in opsonic cultures with P. lophurae in contact 1 hour with hyperimmune or normal test serum.*

Test serum	Culture number	Phagocytic index	% of macrophages		% of macrophages with erythrophagocytosis*		
			With pigment	With parasites	0**	±**	+++**
Hyperimmune	235	0.22	46	54	5	79	16
	236	0.20	42	58	19	78	3
	Mean	0.21 ± 0.01					
Normal	237	0.11	70	30	53	47	0
	238	0.16	70	30	59	41	0
	Mean	0.13 ± 0.01					

* Erythrophagocytosis involved both normal and parasitized red cells.

** 0 = no erythrophagocytosis observed; ± = slight erythrophagocytosis observed; +++ = extensive erythrophagocytosis observed.

were studied, and scavenging phagocytosis was compared to stimulated phagocytosis of *P. lophurae*. These reactions are most conveniently observed in cultures in which the parasitized red cells are exposed to serum for only 1 hour, as explained in experiment 5.

The hyperimmune serum was from the same donor which supplied the serums for cultures 195 and 215 in experiments 11 and 12, respectively, and was collected 75 days after the bird's 6th super-infection with *P. lophurae*. The normal serum was from the donor which supplied the serums used in cultures 212, 233 and 234 in experiments 11 and 12.

The antigen was collected during the acute rise of an initial infection with *P. lophurae* (50 parasites in 100 red blood cells).

Two cultures each were exposed to parasitized red cells in normal and in hyperimmune serum. Fig. 12 and 13 in Plate 1 represent macrophages exposed to normal and hyperimmune serums, respectively.

Agglutination of the supernatant cells, the adhesion of cells to the cover-

slip, in a comparison of the pooled normal with the pooled hyperimmune indices, the value of "t" was 3.9.

The data on scavenging as opposed to stimulated phagocytosis of parasitic material and on erythrophagocytosis, which are presented as in experiment 8, indicate that hyperimmune serum stimulated the phagocytosis of pigmented parasites as opposed to the scavenging of pigment and cellular debris alone, as well as increased the amount of erythrophagocytosis of both normal and infected red cells by individual macrophages and the number of macrophages engaged in this activity.

Thus, the same immune reactions were demonstrated with *P. lophurae* in antilophurae serum as were seen for *P. gallinaceum* in antigallinaceum serum. The reactions were quantitatively more marked in the case of *P. gallinaceum*—perhaps because the antigallinaceum hy-

perimmune serum was collected 12 days after the last superinfection, whereas the antilophuræ serum in the present experiment was collected 75 days after the last superinfection.

6. *The opsonic test with P. lophuræ hyperimmune or normal test serums, collected at closely spaced intervals after a given superinfection of hyperimmune chickens: experiment 14.*—The purpose of this experiment was to study the fluctuations in the opsonic titer in chickens hyperimmunized against *P. lophuræ* for a month following a given superinfection.

Serums were collected just before a given superinfection and every 2 or 3 days for 27 days thereafter from 2 chickens with the following histories: Chicken 3088, an adult cock, was bled 104 days after its sixth superinfection and 12 times after its seventh superinfection. The superinfection was such that 410 parasites per 10,000 red cells were counted in the chicken's blood immediately afterwards and none was found 2 days later. This rapid fall in the number of circulating parasites shows a high degree of immunity to superinfection. Chicken 3093, an adult cock, was bled 106 days after its fifth superinfection and 12 times after its sixth superinfection. The superinfection was such that 320 parasites in 10,000 red cells were counted in its blood immediately afterwards and none was found 2 days later. This chicken, therefore, also had a high degree of immunity to superinfection. Chicken 3095, a normal adult cock, was bled during the same month and at similar intervals.

The antigen was obtained during the acute rise of an initial infection with *P. lophuræ* (40 parasites in 100 red blood cells) and was diluted 1:5 with modified Tyrode's solution.

The cultures were carried through in the usual manner.

The cell suspensions all agglutinated during incubation in the hyperimmune serums, but not in the normal serums.

The phagocytic indices obtained in this experiment are shown in Graph 4. They were consistently low with the serums from normal chicken 3095. On the other hand, they were significantly high with the serums from hyperimmune chicken 3088. The slight drop in

titer immediately after superinfection was probably due to the binding of some of the circulating antibody by the inoculated antigen and was rapidly followed by a progressive rise in opsonic titer which reached a peak 7 days after superinfection. Thereafter, the titer fell irregularly and by the 28th day had reached the level before superinfection. This index (1.15 clumps), however, was still significantly higher than the pooled normal control index (0.26 clump). The indices with the serums from hyperimmune chicken 3093 were significantly different from the pooled normal control index only during the period of 7 to 12 days after superinfection. Thus, whether the amount of circulating opsonin produced by an immune chicken after superinfection was great or small, its titer was highest about a week after a given superinfection.

Although all cultures had received identical leucocyte inoculums, fewer macrophages were seen in the stained preparations of all the cultures exposed to hyperimmune serum than in the normal controls. This fact may be due to the stimulated phagocytic activity of macrophages exposed to hyperimmune serums because engorged macrophages tend to round up, thereby reducing their contact with the coverslip and increasing their chance of being rinsed off. The phagocytic indices of all cultures exposed to hyperimmune serum should, therefore, probably be higher, since the most actively phagocytic cells would be the most likely to become spherical.

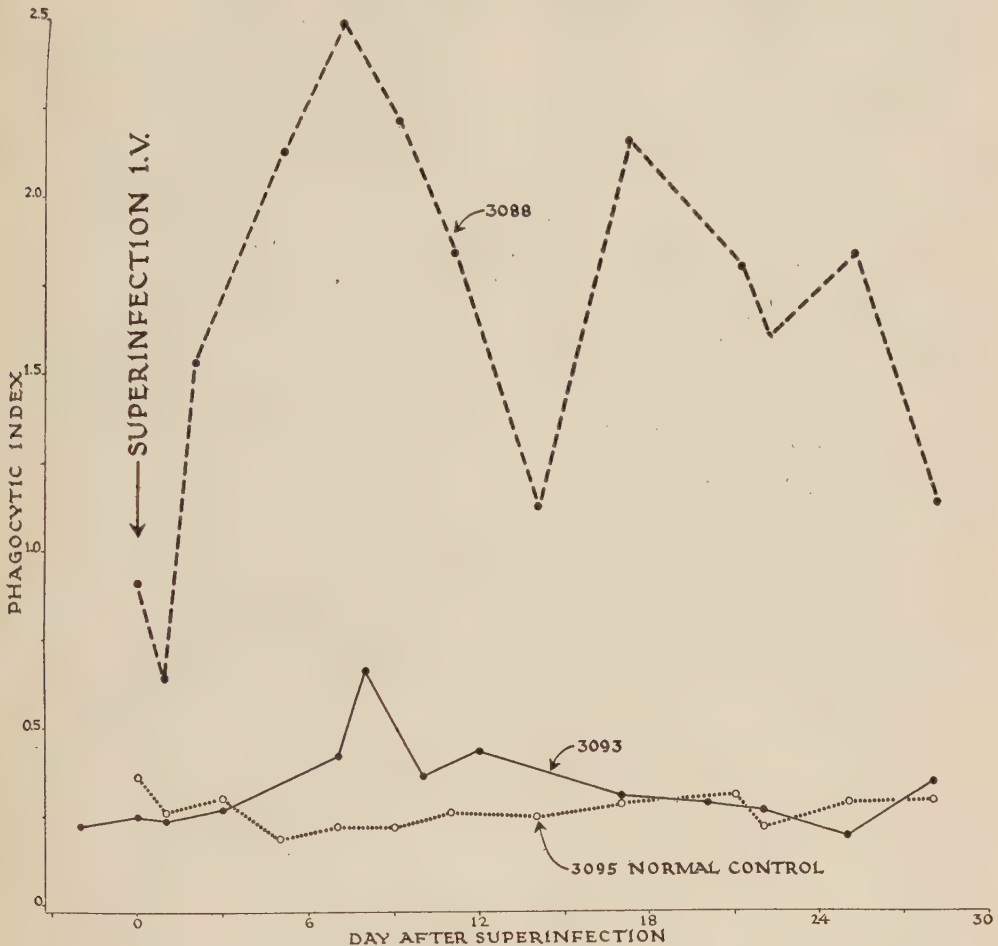
DISCUSSION

Although the study of phagocytosis in vitro can be standardized with respect to such variables as the time of extracting antigen and serum from the host, the amount of antigen, the age of the macrophages and the effective period of phagocytosis, further work is needed before

the present results can be directly correlated with in vivo findings.

The most striking results obtained in the present study of phagocytosis in

cytose free pigment and other debris. In the presence of serum taken after several superinfections, but not after the reduction of the initial infection to



GRAPH 4.—Phagocytic indices in macrophage cultures containing normal (chicken 3095) or hyperimmune serum (chickens 3088 and 3093) and *P. gallinaceum*-infected red cells collected during the acute rise of an initial infection. The cultures were incubated 4 hours with parasites and 20 hours without parasites.

Note that the phagocytic index was consistently low in the cultures containing normal serum, was slightly increased for a few days in the cultures containing hyperimmune serum from chicken 3093, and was high in the cultures containing hyperimmune serum from chicken 3088. Also note that the highest phagocytic indices in the superinfected chickens 3088 and 3093 occurred about a week after superinfection.

tissue culture macrophages are as follows: In the presence of normal serum, or of serum taken during the acute initial infection, the macrophages phago-

latency, there is a stimulated phagocytosis which indicates that infected and uninfected red cells are opsonized. In addition to opsonization, such hyper-

immune serum produces other antibody effects such as agglutination and the adhesion of the red cells to glass surfaces and to macrophages, all of which involve both infected and uninfected red cells.

The observed difference between scavenging phagocytosis in the presence of normal serum and stimulated phagocytosis in the presence of hyperimmune serum in vitro is in line with the observations of Taliaferro and Cannon (1936) on *P. brasilianum* in vivo. They described 2 types of phagocytosis: the sluggish phagocytosis of natural immunity representing the clearing away of debris of parasites dying from other causes, and the more efficient process of acquired immunity representing the active capture of parasites by the macrophages, aided, possibly, by an antibody. The relative unimportance of phagocytosis during the acute rise of malarial infections, and its crucial importance in acquired immunity was further emphasized by Gingrich (1941) for *P. cathemerium* and *P. relictum*.

The tendency of opsonized blood to adhere to macrophages in vitro parallels a similar process in vivo, as described by Taliaferro and Cannon (1936). These authors observed that immunity to *P. brasilianum* involved the filtering of parasitized red cells in the Billroth cords of the spleen, and they ascribed this result to an affinity between the parasitized cells and the splenic macrophages. In the blood-filtering organs not only would circulating opsonin act on the parasitized blood, but the macrophages themselves, which are presumed to produce the antibody, would probably contain an additional amount of opsonin. While circulation at any point in the spleen is sluggish, blood cells with or without parasites can be bound to the macrophages. Later, even when the circulation is rapid, the macro-

phages can ingest such material, since it has been shown in the present work that violent currents in the surrounding fluid are unable to dislodge erythrocytes adhering to macrophages.

It must be emphasized that all the antigen-antibody reactions observed in the present work involved uninfected as well as infected red cells. Not only does this apply to phagocytosis and to the adhesion phenomena, but also to the efficient absorption of antibody from hyperimmune serum by normal red cells.

There are two likely explanations for the action of hyperimmune serum against both normal and parasitized red cells. The first is that this action is due to an immune isoagglutinin which bears no relation to the parasite but acts on the normal constituents of both infected and noninfected red cells. The isoagglutinin would result from the introduction of antigenic factors, foreign to the immunized chicken, in the large doses of parasitized blood repeatedly administered during superinfection. Evidence for such immune isoagglutinins in chickens is given by Todd (1930a; 1930b; 1935). The production of immune isoantibodies to homologous red cells in other animal species has been repeatedly described. Ehrlich and Morgenroth (1900) observed them in goats, Todd and White (1910) in oxen, Thomoff (1930) in horses and mules, Levine and Landsteiner (1931) in rabbits and Frenzel and Szymanowski (1934) in swine. The occurrence of such an immune isoagglutinin might explain why, in the present study, opsonins were found only in chickens hyperimmunized by repeated superinfections with parasitized blood from other chickens.

A second possibility is that the parasite contains some antigen in common with the normal red cell and hence incites the production of an antibody

which reacts with both the parasite and the red cell. Essentially this explanation has been given by Oliver-Gonzalez and Torregrossa (1944), who reported increased titers of α and β isoagglutinins in patients after repeated attacks of *P. vivax* or *P. falciparum*. Their data preclude the view that red cell antigens released during the attacks could have been responsible, since such increases occurred only in isoagglutinins for which no corresponding antigens were present in the host red cells. Since the authors had previously demonstrated helminth antigens capable of inhibiting α and β isoagglutinins, they attributed the increased isoagglutinin titers in human malaria to similar postulated antigens common to human red cells and to plasmodia. The view that plasmodia and normal red cells contain some common factor is supported by the work of Heidelberger and Mayer (1944) who showed that an antigen prepared from normal human red cells fixed complement when combined with serum from chronic relapsing vivax patients.

Unless the concentration of the immune factor in the circulating blood is too low to produce visibly demonstrable effects, neither of these interpretations explains the fact that the red cells of the immunized host are not all opsonized and phagocytosed in great numbers by the macrophages. The ingestion of numerous unparasitized red cells in the *P. brasilianum* infections described by Taliaferro and Cannon (1936) may, however, reflect such an opsonization.

Determination of the nature of the antigen or antigens producing the observed malarial antibodies is complicated by the intimate relationship between plasmodia and erythrocytes. The two interpretations which appear most likely to the author, viz., the possibilities that the antigen or antigens are normal constituents of red cells or are

factors common to plasmodia and red cells, have been discussed above. Stratman-Thomas and Dulaney (1940) suggested that either the parasite or the stroma of the parasitized red cell could be the source of the antigen. The parasite-red-cell combination may produce a soluble antigen which is released into the circulating plasma. Eaton (1939) described a soluble antigen in the serum of *P. knowlesi*-infected monkeys. This serum, injected into normal monkeys, gave rise to the production of complement-fixing antibodies. It is conceivable that the cells used in the superinfecting doses may have been antigenic in the homologous host if they were altered because they were parasitized. Thus, Thomson (1924) suggested as a possible mechanism of blackwater fever the production of a hemolysin against erythrocytes which were so denatured by *P. falciparum* that they were antigenic in the homologous host.

The nature of the immune body associated with recovery and immunity to superinfection may be different in the avian and primate malarias. Thus, all of the present work indicates the fundamental action of an isoantibody operative against both infected and uninfected red cells. In marked contrast to this nonspecific type of reaction is the agglutination of *P. knowlesi* in antiserum derived from the rhesus monkey, as described by Eaton (1938). He found that agglutination was so specifically antiplasmodial that it involved only those infected cells containing large parasites and not those containing smaller ones. This difference between the types of antibody produced in avian and primate malarias may, however, be more apparent than real, since Lack (1942) has described an intermediate situation, in which the intravascular agglutination of *P. catheimerium* at first involved only infected cells, but later in

the infection involved both infected and uninfected erythrocytes.

In the present work, the opsonin titers in some of the birds superinfected with *P. lophurae* were considerably higher than any of the opsonin titers obtained against *P. gallinaceum*. No matter what the nature of the antibody involved, this finding corroborates the fact (Taliaferro, personal communication) that chickens are less easily protected against *P. gallinaceum* than against *P. lophurae* by the inoculation of homologous immune serum.

SUMMARY

An opsonic test has been developed with macrophages grown in vitro from blood agranulocytes of chickens. Red blood cells from chickens having acute initial infections with *Plasmodium gallinaceum* and *Plasmodium lophurae* served as antigens, and serums from normal, homologous immune (initial latent infection) and homologous hyperimmune (after repeated superinfection) chickens served as test serums. Complement was not essential.

The functional capacity of tissue culture macrophages grown from chicken and rabbit blood and the sensitivity of the test were demonstrated with pneumococci and antipneumococcus opsonic serum diluted 1:1000. Many pneumococci were phagocytosed in immune serum, whereas negligible numbers were phagocytosed in normal serum. In addition, agglutination of the cocci and their adhesion to macrophages were observed in dilute immune serum and not in normal serum.

Circulating antibody against normal and parasitized red cells from homologous infections was demonstrated in undiluted hyperimmune serums from chickens superinfected with *P. gallinaceum* and *P. lophurae*, but was not found in immune serums from birds re-

covering from acute initial infections. Its presence was determined by the phagocytic activity of the macrophages and by the agglutination of normal and parasitized red cells and their adhesion to macrophages and to glass surfaces. In general, macrophages in normal serum tended to take up cellular debris and free malarial pigment, whereas in hyperimmune serum they tended to ingest whole parasites, whole uninfected red cells and whole parasitized red cells. Phagocytic indices of undiluted hyperimmune serums were 2 to 9 times as high as the normal controls for antilophurae serums and 2 to 4 times as high as the normal controls for antigallinaceum serums. These opsonic titers were of a lower order than those attained with antipneumococcus serum.

The antibody in antigallinaceum hyperimmune serum was more efficiently absorbed by normal red cells than by an equivalent quantity of homologous parasitized red cells. This finding may indicate that the absorbing material is a constituent of the normal red cell and is partially destroyed in the parasitized cell.

P. gallinaceum-infected red cells were opsonized not only in vitro but were opsonized in vivo under certain conditions. Thus, the phagocytic index of parasitized blood in normal serum was higher when the antigen was collected around the time of the crisis than when it was collected during the early acute rise or just after the crisis.

The amount of phagocytosis in the presence of normal serum varied inversely with the amount of antigen when the latter was collected at the peak of the infection and during the immediately ensuing crisis. The basis for this relationship is not understood, but it may be due to the dilution of some nonspecific factor present at this time. It did not hold when the antigen

was taken at other stages of the infection.

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PLATE.—Macrophages exposed to pneumococci or to *P. lophuræ* in normal or homologous hyper-immune serum.

All drawings were made by Miss Esther Bohlman with the aid of a camera lucida and are $\times 1220$.

Fig. 1.—A rabbit macrophage exposed for 1 hour to pneumococci in normal rabbit serum (1:1000) showing no phagocytosis.

Vacuolated cytoplasm and lacy pseudopods were typical.

Fig. 2, 3 and 4.—Rabbit macrophages exposed for 1 hour to pneumococci in homologous rabbit anti-serum (1:1000) showing marked phagocytosis of pneumococci by the macrophages. Their position in vacuoles distinguish ingested from adhering cocci.

Note the swelling of many ingested cocci and the variations in the intensity with which they took the stain, suggesting that they were being digested by the macrophages. Fig. 4 also illustrates the agglutination of pneumococci around macrophages and their adhesion to macrophages. The elongated, streamer-like form of the agglutinated mass was typical.

Fig. 5.—A chicken macrophage exposed for 1 hour to pneumococci in normal rabbit serum (1:1000) showing sluggish phagocytosis.

Fig. 6 and 7.—Chicken macrophages exposed for 1 hour to pneumococci in homologous rabbit anti-serum (1:1000) showing marked phagocytosis of the pneumococci with digestion of some of the phagocytosed bacteria.

Note agglutination of the cocci both before and after ingestion, and the matrix (probably of capsular material) surrounding the ingested cocci. The matrix is absent from the normal control (Fig. 5) and also from all of the rabbit macrophages (Fig. 1 through 4). As in Fig. 4, Fig. 7 illustrates the agglutination and adhesion of antigen in dilute antibody.

Fig. 8 and 9.—Chicken macrophages exposed for 4 hours to *P. lophuræ* in normal chicken serum (undiluted), then washed and reincubated in normal chicken serum for 20 hours, showing no phagocytosis in Fig. 8 and sluggish phagocytosis in Fig. 9.

Fig. 10 and 11.—Chicken macrophages exposed for 4 hours to *P. lophuræ* in hyperimmune anti-*lophuræ* serum (undiluted) then washed and reincubated in normal chicken serum for 20 hours showing the average quantity of phagocytosed material in a stimulated macrophage.

Note the remnants of both normal and parasitized red cells in the macrophages.

Fig. 12.—A chicken macrophage exposed for 1 hour to *P. lophuræ* in normal chicken serum (undiluted), showing sluggish phagocytosis of the scavenging type.

Note pigment alone in the macrophage.

Fig. 13.—A chicken macrophage exposed for 1 hour to *P. lophuræ* in hyperimmune anti-*lophuræ* chicken serum, showing stimulated phagocytosis of normal and parasitized erythrocytes and rosette formation involving both normal and parasitized erythrocytes.



